IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of : Dario NERI et al.

Serial No.: 09/194,356 Group Art Unit: 1642

Filed: September 2, 1999 Examiner: Alana M. Harris

For: ANTIBODIES TO ED-B DOMAIN OF FIBRONECTIN, THEIR CONSTRUCTION AND USES

DECLARATION UNDER 37 CFR §1.132

I, Luciano Zardi, declare as follows.

1. I am a co-inventor of the above-identified application. I have a financial interest in this application and any resultant patent.

2. My curriculum vitae is attached, demonstrating my expertise in the field of the invention of this application involving antibodies to fibronectins.

3. I am also the last named author on the publication: Carnemolla, et al., "The Inclusion of the Type III Repeat ED-B in the Fibronectin Molecule Generates Conformational Modifications That Unmask a Cryptic Sequence," The Journal of Biological Chemistry, (1992) Vol. 267, No. 34, pp. 24689-24692. [Carnemolla 1992]. A copy of this publication is attached. I am also a co-author on the Carnemolla et al. publication (J. Cell Biol. 108, 1139-1148 [Carnemolla 1989]) mentioned in the first full paragraph on the first page of Carnemolla [1992]). I am also the sole inventor of EP 344134 which discloses an antibody against a fibronectin, which is named BC-1. (A copy of EP 344134 is also attached.)

4. There are two forms of fibronectin which are of interest here. One of these has a known structure which includes a number of domains known as Type III homology repeats. These are depicted in Figure 2A of the above-identified application. (Copy attached for convenience). In the following, this first kind of fibronectin will be termed FN. The second type of fibronectin at issue here is identical in sequence to FN but additionally contains one

more domain, which is another Type III homology repeat spliced between repeats 7 and 8 of FN. This additional Type III repeat is known as the ED-B domain. This second kind of fibronectin is termed herein, FN-ED-B.

5. When I first produced antibody BC-1, I disclosed it as being specific for the ED-B domain of FN-ED-B. (Carnemolla 1989; EP 344134). I based this, in part, on the fact that BC-1 reacted with FN-ED-B but not with FN. However, it is now scientifically established that my original conclusion was incorrect. The reality is that the antibody BC-1, while being specific for FN-ED-B, in the sense that it does not react with FN, does not react with (i.e., is not specific for) the ED-B domain itself. Rather, when BC-1 binds to FN-ED-B, it reacts with (i.e., binds to) a region of FN-ED-B which is not the ED-B region; rather, it turns out that BC-1 binds to Type III homology repeat 7, a repeat which is adjacent to the ED-B domain.

6. One question we considered concerns how BC-1 is able to bind to homology repeat 7 in FN-ED-B, yet does not bind at all to FN which also contains homology repeat 7. We have now established the reason for this: when the additional Type III homology repeat ED-B is inserted into FN between the repeats 7 and 8 to form FN-ED-B, the resultant new protein takes on a different conformation in comparison with that of FN (without the additional ED-B domain). In this modified three-dimensional structure (conformation), the region surrounding the ED-B domain is, as might be expected, affected. One of the effects is to change the three-dimensional structure of the repeat 7 in comparison with its three-dimensional structure in the FN molecule. One of the differences is that an epitope (specific protein sequence uniquely bound by an antibody) is exposed in repeat 7 of FN-ED-B which was not exposed (i.e., was hidden or masked or, more scientifically, was "cryptic") in homology repeat 7 of FN.

7. Our studies have also shown that the mechanism underlying these conformational modifications in repeat 7 involves the presence of homology repeat 9. The latter interacts with repeat 7 to mask the epitope at issue when ED-B is lacking.

8. In other words, originally, we (Carnemolla 1989) thought that, because BC-1 required the presence of ED-B in order to bind to FN, BC-1 was binding directly to the ED-B domain. But this turns out to be wrong. Instead, the presence of the ED-B domain in FN is necessary

for BC-1 to bind thereto, but not because ED-B is the site of binding of BC-1 to FN; rather, ED-B is necessary to change the conformation of repeat 7 of FN in order to produce a modified conformation which exposes an otherwise hidden epitope in repeat 7 to which BC-1 will bind.

9. The following summarizes the scientific proof we have developed which proves that BC-1 does not bind directly to the ED-B domain of FN- ED-B but rather binds to its type 7 repeat. It also summarizes the proof we have developed that two of the antibodies of this invention (named CGS-1 and CGS-2), unlike BC-1, are the first antibodies which bind directly to the ED-B domain of FN-ED-B.

10. This scientific proof is contained in Carnemolla et al. 1992 and in Example 3 of the above-identified application. I hereby confirm that the scientific experiments and results reported in Carnemolla 1992 and Example 3 of the above-identified application are true and accurate. A copy of Example 3 of the above-identified application is attached for convenience.

11. As explained in Carnemolla 1992, in order to prove the foregoing conclusions, we fabricated a series of partial fibronectin molecules. These are discussed in Figures 2 and 5 of Carnemolla 1992. For convenience, these data are summarized below in Table 1.

TABLE 1

Number	FN Fragment	Type III Repeats present	BC-1 binds
1	λF7.8.9/2	7,8 and half of 9	-
2	λF7.B.8.9/2	7,B,8 and half of 9	+
3	λF7.8	7 and 8	+
4	λF 7/3.8	one third of 7 and all of 8	-
5	λF7.8/18	7 and 1/18 of 8	+
6	λF7	7	+

12. In binding experiment no. 1 of the table, repeats 7 and 8 and half of repeat 9 ("9/2") were included. As expected from the observations on BC-1 summarized above, there was no

binding by BC-1, because the molecule lacked the ED-B domain. In experiment number 2, the ED-B domain was added between homology repeats 7 and 8 of experiment 1. The presence of the ED-B domain enabled BC-1 to bind to the molecule, as explained above.

13. In experiment number 3, homology repeat 9 was completely eliminated as was the ED-B domain. Nevertheless, the BC-1 antibody still bound to the molecule. This establishes that BC-1 is not binding to the ED-B domain which is no longer present, contrary to what Carnemolla 1989 and EP 344134 said. It also establishes that BC-1 is not binding to the also missing homology repeat 9. It shows that BC-1 is binding to either of repeats 7 and 8, the only two present. Because this antibody does not bind to 7 or 8 when also half of repeat 9 is present (experiment 1), these experiments together show that repeat 9 is involved in preventing binding of BC-1 to repeat 7 or 8 when ED-B is not present.

14. In experiment number 4, two thirds of homology repeat 7 is eliminated ("7/3"). This eliminates the binding of BC-1. This establishes that BC-1 is not binding to homology repeat 8 which is still present but rather, apparently, is binding to part of the two thirds of homology repeat 7 which has been eliminated. In experiment number 5, all of homology repeat 7 is retained and only one eighteenth of homology repeat 8 is present. This confirms that homology repeat 8 is not necessary for binding but homology repeat 7 is. Finally, in experiment number 6, as expected from the other experiments, when the molecule contains only homology repeat 7, BC-1 binds thereto.

15. The conclusion from this Table summarizing data from Carnemolla 1992 is clear: BC-1 will only bind to FN when the ED-B domain is present (as also established by the earlier data in Carnemolla 1989 and EP 344134), but BC-1 is not binding directly to the ED-B domain in such case; rather, it is binding to an epitope in the homology repeat 7 domain. Moreover, because BC-1 binds to homology repeat 7 in the presence of homology repeat 8 (experiment number 3) but will not bind to homology repeat 7 in the presence of even half of homology repeat 9 (experiment number l) unless the ED-B domain is also present (experiment number 2), these data elucidate the mechanism, discussed above, by which the BC-1 epitope in homology repeat 7 is masked in FN, i.e., due to an effect from homology repeat 9.

16. The following Table II qualitatively summarizes the data in Table 2 of Example 3 of the above-identified application. Omitted are data serving essentially as negative controls.

TABLE 2

Number	CGS-1	CGS-2	BC-1	Description
1 Plasma FN	-	-	-	human plasma FN (no ED-B)
2 WI38VA FN	+	+	+	FN containing ED-B
3 n110 kD (B-)	-	-	-	repeats 2-11 (no ED-B) (FIG 2A)
4 n120 kD (B+)	+	+	+	repeats 2-11 (with ED-B) (FIG 2A)
5 rec FN7B89	+	+	+	repeats 7,B, 8, 9
6 rec FN 789	-	-	-	repeats 7,8, 9
7 rec ED-B	+	+	-	ED-B

17. The first two experiments merely confirm the foregoing observations, as do the third and fourth experiments. These show that BC-1 only binds to full length FN (experiments 1 and 2) or repeat regions 2-11 of FN (experiments 3 and 4) when the ED-B domain is present. The same is true of the antibodies of this invention, CGS-1 and -2. Experiments 5 and 6 reflect the fact that BC-1 will bind to homology repeat 7 as long as the EDB domain is present but will not do so when it is missing and repeat 9 is present. CGS-1 and CGS-2 also bind to the construct of experiment 5 because it contains ED-B but do not bind to the experiment 6 construct because it lacks ED-B. Finally, reflecting the essential difference between the prior art BC-1 antibody (which binds to repeat 7) and two of the antibodies of this invention, CGS-1 and -2 (which directly bind to ED-B), BC-1 fails to bind to the ED-B domain per se whereas CGS-1 and CGS-2 do bind to it.

CONCLUSION: The prior art antibody BC-1 binds to FN-ED-B by reacting specifically with the homology repeat 7 of FN-ED-B and not by binding to the ED-B domain itself. In

contrast, antibodies of this invention such as CGS-1 and CGS-2 are the first antibodies to bind to FN-ED-B directly by reacting with (binding to) the ED-B domain itself.

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

November 18, 2003
Date

Dr. Luciano Zardi

LUCIANO ZARDI
Curriculum Vitae

PERSONAL

Date and place of birth	April 14th, 1943, Annicco (Cremona)
Marital status	Married 1968, a son 1973
Military	November 1963 – February 1965
Present address	Via San Fortunato, 3 Camogli, Genoa, Italy

EDUCATION

1971 Ph.D degree in Biological Sciences, University of Milan
1977 Specialization in Biological Chemistry, University of Milan

APPOINTMENTS

August 2003 to present	Director, Unit of Innovative Therapies, Istituto Scientifico Giannina Gaslini
2003	President, Italian Society of Applied Biosciences
1980 to August 2003	Director, Laboratory of Cell Biology, Istituto Nazionale per la Ricerca sul Cancro (National Cancer Research Institute of Genoa), Largo Rosanna Benzi, 10, 16132, Genoa, Italy.
1982 to present	Professor on annual contract, Developmental Biology, University of Genoa.
Sept. 1997 – March 1999	Member of "National Committee for Biological Sciences and National Research Council (CNR)".
Nov. 1980 – July 1981	Visiting Scientist, Institute of Biochemistry, University Lausanne, Swiss.
Jan/Feb '78 & July/Aug.'80	Visiting Scientist, Wistar Institute, Philadelphia, USA
1977 to present	Responsible of the grants from National Council for Research (CNR), AIRC, European Community (from 1997 Biotechnology) and Ministry for Research (MURST) and Health.
1977 – 1989	Associate Professor in Experimental Oncology in graduate course of Oncology
1975 – 1976	Consultant International Agency for Research on Cancer WHO) Lyon, France.
1975 – 1980	Senior Researcher, Institute of Oncology, University of Genoa, Italy.
1972 – 1975	Research Associate, Temple University of Philadelphia, USA.
1962 – 1972	Research Fellow, Institute of Medical Semeiotic, Pathology and Medical Clinic, University of Milan, Italy.

AWARDS

2000	Winner with Prof. Dr. Dario Neri, of "ABBOTT Award 2000"

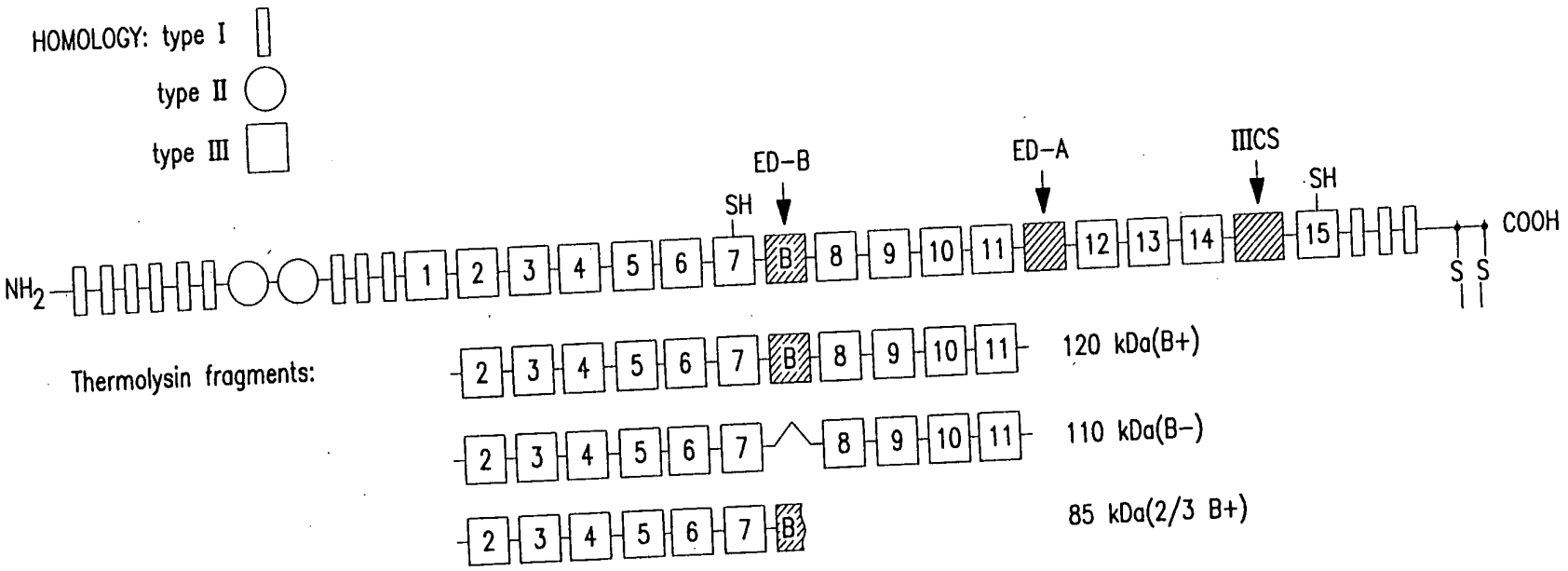
HOMOLOGY: type I
type II
type III
ED-B
ED-A
IIICS
SH
SH
COOH
NH2
S S
Thermolysin fragments:
120 kDa(B+)
110 kDa(B-)
85 kDa(2/3 B+)

FIG. 2A

XP002042101

THE JOURNAL OF BIOLOGICAL CHEMISTRY
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Vol. 267, No. 34, Issue of December 5, pp. 24689-24692, 1992
Printed in U.S.A.

The Inclusion of the Type III Repeat ED-B in the Fibronectin Molecule Generates Conformational Modifications That Unmask a Cryptic Sequence*

(Received for publication, July 10, 1992)

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We have previously reported an anti-fibronectin monoclonal antibody (mAb) (BC-1) which reacts with an ED-B-containing β-galactosidase-fibronectin fusion protein but not with an identical β-galactosidase-fibronectin fusion protein in which the ED-B sequence is omitted. In further experiments aimed at localizing more precisely the epitope recognized by this mAb, we demonstrate that 1) the mAb BC-1 is indeed specific for ED-B-containing fibronectin (FN) molecules even though the epitope recognized by this mAb is localized on the type III homology repeat 7 (the one which precedes the ED-B sequence) and 2) in fibronectin molecules lacking the ED-B sequence, this epitope is masked. We further demonstrate that, to mask the epitope recognized by the mAb BC-1, the presence of at least half of the FN type III homology repeat 9 is necessary. We also report the production of the mAb IST-6 which recognizes only FN molecules in which the ED-B sequence is lacking. These data clearly demonstrate that the presence of the ED-B sequence within FN molecules generates conformational modification in the central part of the molecules that unmasks previously cryptic sequences and masks others.

Fibronectins (FNs)[1] are high molecular mass adhesive glycoproteins present in the extracellular matrix and in body fluids. These molecules are involved in different biological phenomena such as the establishment and maintenance of normal cell morphology, cell migration, hemostasis and thrombosis, wound healing, and oncogenic transformation (for reviews, see Alitalo and Vaheri (1982), Yamada (1983), Hynes (1985), Ruoslahti (1988), Hynes (1990), Owens *et al.* (1986)).

FN polymorphism is due to alternative splicing patterns in three regions (IIICS, ED-A, and ED-B) of the single FN primary transcript (see Fig. 1) as well as to post-translational modifications. The alternative splicing of the FN pre-mRNA is regulated in a cell-, tissue-, and developmentally specific manner (see above-mentioned reviews and references therein). Furthermore, it has been demonstrated that the splicing pattern of FN pre-mRNA is deregulated in transformed cells and in malignancies (Castellani *et al.*, 1986; Borsi *et al.*, 1987; Vartio *et al.*, 1987; Zardi *et al.*, 1987; Barone *et al.*, 1987; Carnemolla *et al.*, 1989; Oyama *et al.*, 1989, 1990; Borsi *et al.*, 1992). In fact, the FN isoforms containing the IIICS, ED-A, and ED-B sequences are expressed to a greater degree in transformed human cells and in tumor tissues than in their normal counterparts. In particular, the FN isoform containing the ED-B sequence, which, with some very rare exceptions, is undetectable in normal adult tissues, has a much greater expression in fetal and tumor tissues as well as during wound healing (Norton and Hynes, 1987; Schwarzbauer *et al.*, 1987; Gutman and Kornblihtt, 1987; Carnemolla *et al.*, 1989; ffrench-Constant *et al.*, 1989; ffrench-Constant and Hynes, 1989, Laitinen *et al.*, 1991). The ED-B oncofetal domain, a complete type III homology repeat composed of 91 amino acids and coded for by a single exon, is the most conserved FN region with 100% and 96% homology with rat and chicken FN, respectively (Norton and Hynes, 1987); Zardi *et al.*, 1987). This could indicate either a more recent evolution of the ED-B sequence, with less time to diverge, or a more stringent requirement due to some unknown function(s) performed by this sequence. While the alternative spliced sequence IIICS contains a cell type-specific cell binding site, the biological functions of the ED-A and ED-B are still a matter of speculation (Humphries *et al.*, 1986).

We describe two monoclonal antibodies, BC-1 and IST-6, which are specific for FN isoforms containing and not containing the ED-B sequence, respectively. Given this specificity, we assumed that the epitope recognized by the mAb BC-1 was localized within the ED-B sequence (Carnemolla *et al.*, 1989). However, we now demonstrate that this epitope is localized within the type III repeat 7 (which precedes the ED-B) and that it is cryptic in FN molecules lacking the ED-B, while it is unmasked in molecules containing this sequence.

MATERIALS AND METHODS

Cell Lines, Monoclonal Antibodies (mAbs), Radioimmunoassay, and Purification of FN and Its Proteolytic Fragments—The cultured normal human fibroblast cell line GM-5659 (from non-fetal skin) and the SV40-transformed WI-38-VA cell line were purchased from NIGMS, Human Genetic Mutant Cell Repository (Camden, NJ) and from the American Tissue Type Culture Collection, respectively. The mAb IST-6 was prepared as described using splenocytes from mice immunized with FN purified from human plasma (Zardi *et al.*, 1980). The mAb BC-1 has been described previously (Carnemolla *et al.*, 1989). FNs were purified from human plasma and from the conditioned medium of the various cell lines using a modification (Zardi *et al.* (1980)) of the procedure of Engvall and Ruoslahti (1977). The radioimmunoassay experiments, using purified FNs or FN fragments, were carried out as reported by Zardi *et al.* (1980). The 120-kDa (containing the ED-B) and 110-kDa (lacking the ED-B) FN domain 4 isoforms (see Fig. 1) were purified from thermolysin digests as previously described (Zardi *et al.*, 1987); Borsi *et al.*, 1991).

cDNA Clones and Fusion Proteins—cDNA clones λF2 and λF6c, obtained as previously reported (Carnemolla *et al.*, 1989), cover the

* This study was partially supported by Associazione Italiana per la Ricerca sul Cancro (AIRC) funds and Consiglio Nazionale delle Ricerche (CNR), "Progetto finalizzato: applicazioni cliniche della ricerca oncologica." The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] The abbreviations used are: FN, fibronectin; mAb, monoclonal antibody; PAGE, polyacrylamide gel electrophoresis.

ty, .II homology repeats 7, 8, and half of the 9 (from residues 1138 to 1380) (Kornblihtt *et al.*, 1985). However, the ED-B sequence was included in the λF2 clone while it was omitted in the λF6c clone. In order to facilitate the reading of the manuscript, we have here named these clones according to the type III repeats or fractions of type III repeats they contain: λF2 = λF7.B.8.9/2; λF6c = λF7.8.9/2 (see Fig. 1A). The cDNA clones λF7.8/18 and λF7 were obtained from the λF7.8.9/2 FN insert by digestion with *Bsp*M1 and PV2, respectively. The λF7.8/18 cDNA clone includes the type III homology repeat 7 and few amino acids of the 8 (from residues 1138 to 1239), while the λF7 cDNA clone is made up of repeat 7 (from residues 1138 to 1234). The inserts of the clones λF7.8 and λF7/3.8 were obtained from polymerase chain reaction amplification of the λF7.8.9/2 using λgt11 primers (New England Biolabs) and appropriate oligonucleotides. λF7.8 included the type III homology repeats 7 and 8 (from residues 1138 to 1318) while the clone λF7/3.8 included one-third of the 7 and the 8 (from residues 1167 to 1318). PCR reactions were performed for 35 cycles (1 min at 94 °C, 1 min at 48 °C, and 1 min at 68 °C) in a final volume of 100 μl containing 50 mM KCl, 10 mM Tris-Cl, pH 8.3, 1.5 mM $MgCl_2$, 0.01% (w/v) gelatin, 200 mM concentration of each dNTP, 100 pmol of each oligonucleotide, 5 units of *Taq* DNA polymerase (Amplitaq, Perkin-Elmer Cetus), and 10 ng of purified λF7.8.9/2 DNA as template. All cloning and subcloning procedures were carried out, using the expression vector λgt11 phage, according to Sambrook *et al.* (1989), and each DNA insert was analyzed using Sequenase version 2.0 DNA sequencing kit (United States Biochemical Corp.). The β-galactosidase-FN fusion proteins were prepared as previously described (Carnemolla *et al.*, 1989), and their immunoenzymatic reaction with mAbs IST-6 and BC-1 was carried out using a ProtoBlot immunoscreening system kit purchased from Promega Biotec. SDS-PAGE and immunoblotting were carried out as previously reported (Carnemolla *et al.*, 1989).

RESULTS AND DISCUSSION

Fig. 1A depicts the domain structure of a human FN subunit. We have tested the mAbs BC-1 and IST-6 with fibronectins containing different percentages of ED-B-containing molecules. We have previously reported that the ED-B-containing FN is absent in plasma and present in a very low percentage in FN from cultured normal skin fibroblasts, while it is present in a high percentage in FN from SV40-transformed WI-38-VA cells (Borsi *et al.*, 1992; Carnemolla *et al.*, 1989). Fig. 1 shows the results obtained by radioimmunoassay experiments using the mAbs BC-1 and IST-6 on FNs from these three different sources. The mAb BC-1 does not react with plasma FN and barely reacts with FN from normal skin fibroblasts, while it reacts strongly with FN from the SV40-transformed WI-38-VA cell line. On the contrary, the mAb IST-6 gives a strong reaction with FNs from plasma and normal skin fibroblasts while it shows a weaker reaction with FN from WI-38-VA cells. Fig. 2 shows the results obtained by immunoscreening using the monoclonals BC-1 and IST-6 on lysis plaques generated by the clones λF7.B.8.9/2 and λF7.8.9/2 (Fig. 2). The mAb BC-1 reacts with the fusion protein λF7.B.8.9/2 but not with λF7.8.9/2. On the contrary, the mAb IST-6 does not react with the fusion protein λF7.B.8.9/2 but does with the fusion proteins λF7.8.9/2. Identical results were obtained in immunoblotting experiments after SDS-PAGE using the λF7.B.8.9/2 and λF7.8.9/2 fusion proteins (Fig. 3) and the 120 (ED-B+)- and 110 (ED-B−)-kD

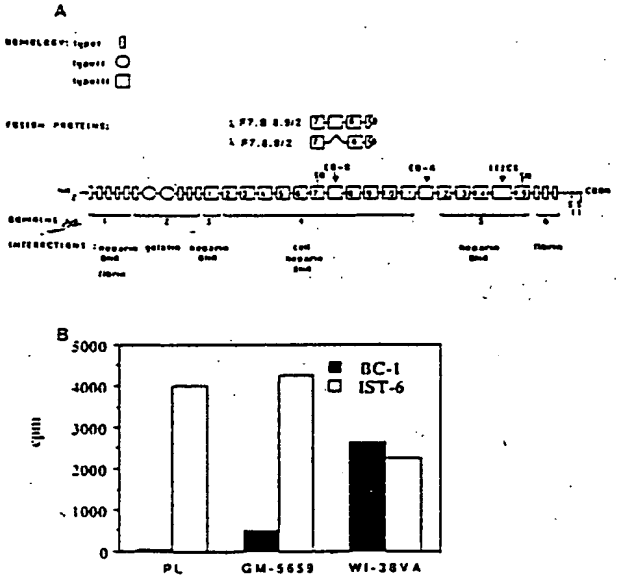

FIG. 1. A, model of the domain structure of a human FN subunit. The IIICS, ED-A, and ED-B regions of variability, due to alternative splicing of the FN pre-mRNA, are indicated. The figure also indicates the internal homologies, macromolecules interacting with the various FN domains, and the β-galactosidase-FN fusion proteins expressed in *Escherichia coli* containing (λF7.B.8.9/2) and not containing (λF7.8.9/2) the ED-B sequence. *B*, radioimmunoassay analysis of plasma FN (*PL*), FN purified from the conditioned media of normal human skin fibroblast cell line, GM-5659, and of WI-38-VA, SV40-transformed embryonic lung fibroblast, using the mAbs BC-1 (*black bars*) and IST-6 (*white bars*).

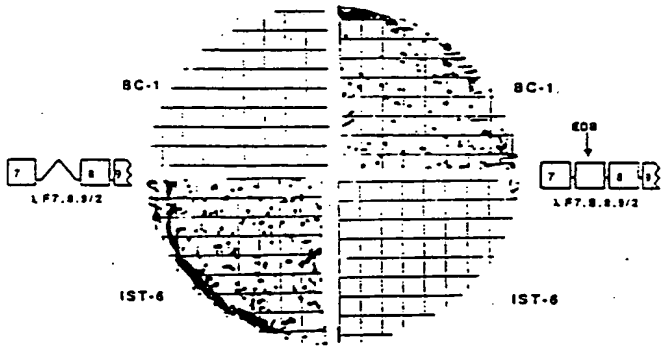

FIG. 2. **λF7.8.9/2 and λF7.B.8.9/2 clones were plated on *E. coli* cells, overlaid with a nitrocellulose filter paper previously saturated with isopropyl β-D-thiogalactopyranoside, and probed with mAbs BC-1 and IST-6.** BC-1 gives positive signals with λF7.B.8.9/2 lysis plaques only. On the contrary, IST-6 gives positive signals with λF7.8.9/2 lysis plaques only.

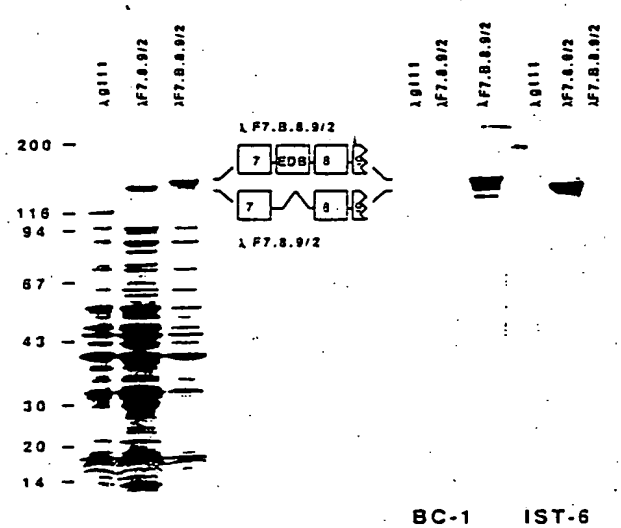

FIG. 3. On the *left*, a 4–18% SDS-PAGE gradient of proteins from *E. coli*-infected by the expression vector λgt11 and by the clones λF7.B.8.9/2 and λF7.8.9/2, respectively. On the *right*, immunoblots using the mAb BC-1 and IST-6, respectively. The values on the *left* indicate the molecular masses, in kDa, of the standards.

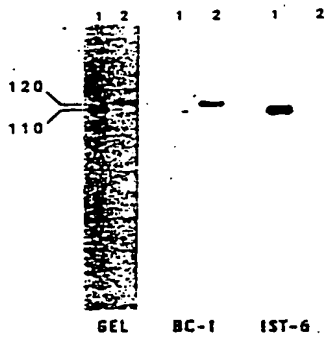

FIG. 4. On the *left*, a 4–18% SDS-PAGE gradient of the purified ED-B− (*lane 1*) 110- and ED-B+ 120-kDa (*lane 2*) domain 4. On the *right*, immunoblots of a similar gel using the mAbs BC-1 and IST-6. The values (in kDa) on the *left* indicate the molecular masses of the two FN fragments.

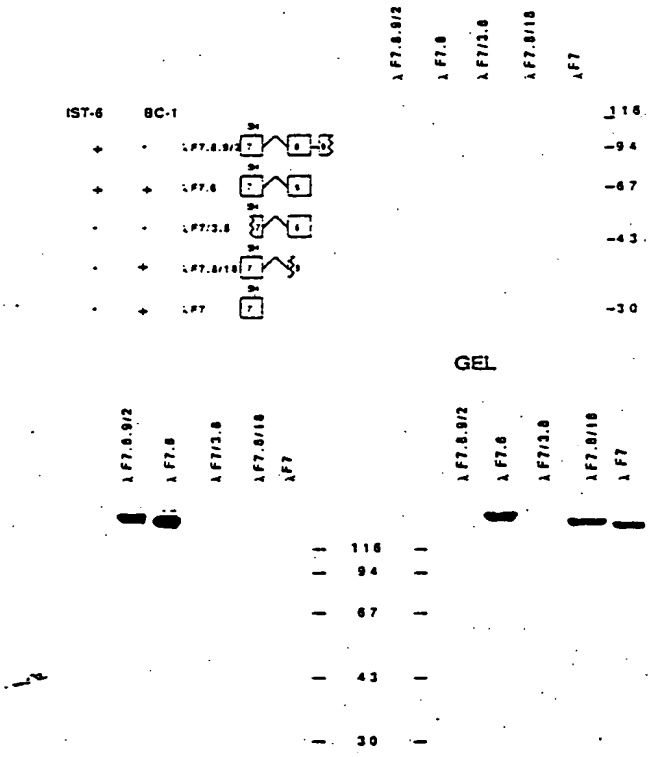

FIG. 5. On the *left* (*top*), the FN sequences contained in the fusion proteins expressed in *E. coli* infected by the clones λF7.8.9/2, λF7.8, λF7/3.8, λF7.8/18, λF7, and the reactivity of these fusion proteins with the mAbs BC-1 and IST-6. On the *right* (*top*), a 4–18% SDS-PAGE of proteins from *E. coli* infected by the clones described above. The values on the *right* indicate the molecular masses, in kDa, of the standards. At the bottom, immunoblots of similar gel using the mAb BC-1 and IST-6. The values in the *middle* of the figure indicate the molecular masses, in kDa, of the standards.

domains 4 (Zardi *et al.*, 1987) with the mAbs BC-1 and IST-6 (Fig. 4). These results demonstrate that BC-1 and IST-6 are specific for FN molecules containing and not containing the ED-B sequence, respectively.

In immunoblotting experiments using different FN-β-galactosidase fusion proteins, we have more precisely localized the epitope recognized by the mAb BC-1. Even though the experiments shown in Figs. 1–4 could suggest that this epitope is localized within the ED-B sequence, since it is specific only for ED-B-containing FN molecules or fragments, the results shown in Fig. 5 demonstrate that it is localized within the type III homology repeat 7 and that the presence of the type III homology repeat 9 makes this epitope cryptic, while the expression of the ED-B sequence unmasks it. In fact, a FN-β-galactosidase fusion protein identical with the λF7.8.9/2 but lacking the repeat 9 reacts strongly with the mAb BC-1. In addition, the β-galactosidase-FN fusion protein λF7 which contains only the repeat 7 also shows the same positive reaction with the mAb BC-1. The mAb BC-1 also reacted with a β-galactosidase-FN fusion protein which contains the repeats 7 and ED-B (data not shown) but did not show any reaction with β-galactosidase-FN fusion proteins containing, respectively, only the repeat ED-B or the repeat 9 (data not shown).

The epitope recognized by the mAb IST-6 was more problematic to localize; in fact, the minimum requirement for a positive reaction with the mAb IST-6 for a FN-β-galactosidase fusion protein was the simultaneous presence of the complete type III homology repeats 7 and 8 (Fig. 5).

The main observations described here are that 1) the mAb BC-1 is specific for ED-B-containing FN molecules even though the epitope recognized by this mAb is not localized within the ED-B sequence and 2) the mAb IST-6 is strictly specific for FN molecules lacking the ED-B sequence. Thus, these two mAbs represent useful reagents to study the distribution of different FN isoforms in different tissues. Furthermore, these data demonstrate that the presence of the ED-B sequence within the FN molecules induces conformational modification in the central part of the molecule which, in turn, leads to the unmasking of a previously cryptic sequence within the type III repeat 7 and to the masking of the epitope recognized by the mAb IST-6. The fact that these structures are detectable also in immunoblotting experiments, after electrophoresis in presence of SDS, indicates that strong interactions between different FN regions take place. It has been reported that the free sulfhydryl group present in the repeat 7 is cryptic when plasma FN is in solution, while it is unmasked in FN bound to solid phase substrates (Narasimhan *et al.*, 1988; Narasimhan and Lai, 1991). However, the mAb BC-1 recognized an epitope in which this cysteine is not involved because, in FN molecules lacking the ED-B, it is cryptic when FN is in solution or when it is bound to solid phase substrates. Furthermore, in the β-galactosidase-FN fusion protein λF7.8.9/2 (see Fig. 5), we have substituted the cysteine present in the repeat 7 with a serine; the reactivity of this mutant with mAbs BC-1 and IST-6 did not show any modification (data not shown). At present, we have no evidence of whether these conformational modifications may influence the biological functions of FN. However, it has been reported that the cell binding site GRGD needs two synergistic regions in order to express its activity, and these regions have been localized within type III homology repeats 8 and 9, respectively (Obara *et al.*, 1988); Nagai *et al.*, 1991). Further studies are needed in order to clarify the structural conformation of this crucial region of the FN molecule and the possible functional modifications induced by the expression of the ED-B sequence.

Acknowledgments—We thank Antonella Gessaga for skillful secretarial assistance and Thomas Wiley for manuscript revision. We are indebted to Prof. L. Santi for his support and encouragement.

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December 5, 1992 VOLUME 267 NUMBER 34

ISSN 0021-9258
JBCHA3 267(34) 24149-24912 (1992)

THE Journal of Biological Chemistry

Published by the American Society for Biochemistry and Molecular Biology

FOUNDED BY CHRISTIAN A HERTER
AND SUSTAINED IN PART BY THE CHRISTIAN A HERTER MEMORIAL FUND

Additions and Corrections

Vol. 267 (1992) 24689–24692

Th inclusion of the type III repeat ED-B in the fibronectin molecule generates conformational modifications that unmask a cryptic sequence.

Barbara Carnemolla, Alessandra Leprini, Giorgio Allemanni, Marco Saginati, and Luciano Zardi

Page 24691, Fig. 5: This figure was reproduced incorrectly. The complete figure and legend are at right:

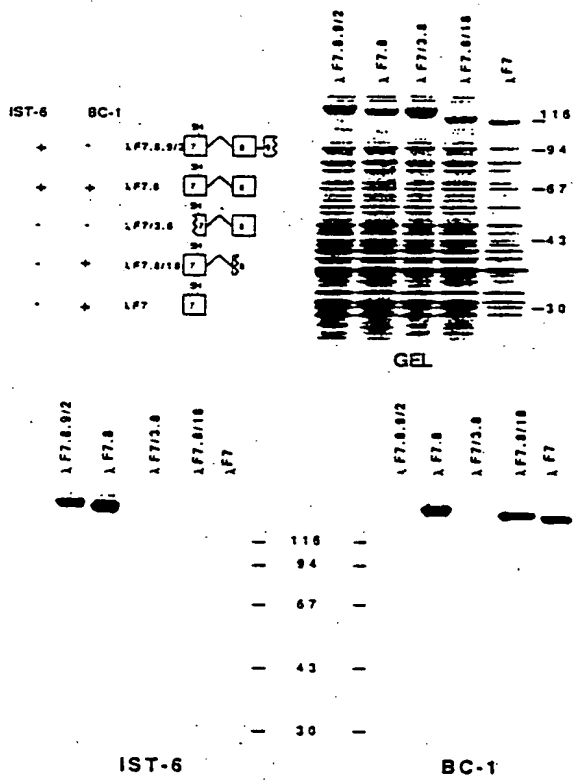

FIG. 5. On the *left* (*top*), the FN sequences contained in the fusion proteins expressed in *E. coli* infected by the clones λF7.8.9/2, λF7.8, λF7/3.8, λF7.8/18, λF7, and the reactivity of these fusion proteins with the mAbs BC-1 and IST-6. On the *right* (*top*), a 4–18% SDS-PAGE of proteins from *E. coli* infected by the clones described above. The values on the *right* indicate the molecular masses, in kDa, of the standards. At the bottom, immunoblots of similar gel using the mAb BC-1 and IST-6. The values in the *middle* of the figure indicate the molecular masses, in kDa, of the standards.

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(19) Europäisch s Patentamt
Eur pean Pat nt Offic
Offic ur péen des brevets

(11) Publication number: 0 344 134 A2

(12) EUROPEAN PATENT APPLICATION

(21) Application number: 89830234.4

(22) Date of filing: 25.05.89

(51) Int. Cl.4: C 07 K 15/00
C 12 P 21/00, C 12 N 5/00,
G 01 N 33/574
//C12N15/00,(C12P21/00,
C12R1:91)

(30) Priority: 26.05.88 IT 2074188

(43) Date of publication of application:
29.11.89 Bulletin 89/48

(84) Designated Contracting States:
AT BE CH DE FR GB IT LI LU NL SE

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Claims for the following Contracting States: ES + GR
The microorganism(s) has (have) been deposited with European Collection of Animal Cell Structures under number(s) 88042101.

(54) **Monoclonal antibody specific to a fibronectin sequence expressed in transformed cells, hybridoma secreting said antibody and use of said monoclonal antibody in the diagnosis of tumors.**

(57) A monoclonal antibody specific to the ED-B sequence of fibronectin expressed in transformed cells. The antibody, which is obtained from a hybridoma deriving from the fusion of immunized mice spleen cells using fibronectin from the culture medium of SV40-transformed embryonic human lung cells with mouse myeloma cells, is useful for the diagnosis of tumors.

EP 0 344 134 A2

Des ription

MONOCLONAL ANTIBODY SPECIFIC TO A FIBRONECTIN SEQUENCE EXPRESSED IN TRANSFORMED CELLS, HYBRIDOMA SECRETING SAID ANTIBODY AND USE OF SAID MONOCLONAL ANTIBODY IN THE DIAGNOSIS OF TUMORS

The present invention relates to a specific monoclonal antibody to a protein sequence coded for by the exon ED-B of human fibronectin, to an hybridoma secreting said antibody and to the use of said antibody in the diagnosis of tumors.

Fibronectins are a class of high molecular weight glycoproteins present in soluble form in plasma and other body fluids and in insoluble form in the extracellular matrices. Fibronectin molecules are involved in various biological phenomena, such as the maintenance of normal cell morphology, cell migration, hemostasis and thrombosis, wound healing and oncogenic transformation. Fibronectin (FN) is known to consist of a mixture of molecules of different structures (isoforms) whose characteristics vary depending on the originary tissue. FN from transformed human cell - cultures has an isoform composition very different from the normal counterpart. As a matter of fact, transformed cells produce remarkable amounts of a FN isoform (β-FN) which is poorly present in normal cells. Recently we demonstrated that this FN form derives from a differential splicing pattern of the FN MRNA precursor which leads in transformed cells to a high-level expression of the ED-B exon which, on the contrary, is poorly expressed in normal cells (EMBO J., Volume 6, pages 2337-2342, 1987).

The monoclonal antibody according to the present invention allows the analysis of the isoform composition of FN in human tissues obtained from biopsies and therefore is suited for many important diagnostic and/or therapeutic uses.

The antibody of the invention, due to its high affinity and specificity, provides an ideal reagent for evidencing the oncofetal FN isoform in biopsias of human tissues.

According to a further object of the invention, an hybridoma is provided obtained by fusing spleen cells from immunized mice, using FN derived from a culture medium of SV40-transformed embryonic cells with myeloma cells from P3U1 mice.

FN which had been purified from the culture medium of SV40-transformed Wi-38 embryonic human lung cells and defined Wi-38Va13, was used to immunize the animals. The used immunization and fusion techniques are the conventional ones and they will be described in detail in the Example hereinafter. The antibody-secreting hybridoma of the invention was deposited on April 21, 1988 at the European Collection of Animal Cell Cultures, Porton Down, Salisbury, Great Britain, under the n° 88042101.

The monoclonal antibody of the invention was found to be highly specific and selective for an epitope present in the ED-B sequence of FN from Wi-38Va13 cells, as it could be demonstrated using the Western Blot technique, with plasma FN or thermolysin-digested FN from Wi-38 or Wi-38Va13 cells. The monoclonal antibody of the invention does not react neither with plasma FN nor with FN from Wi-38 normal human fibroblasts.

In order to evaluate the applicative possibilities of the antibody of the invention, the distribution epitope recognized by the antibody was investigated in a variety of fetal and adult tumoral and normal tissues.

The results from the immuno-histochemical analysis of a variety of fetal and adult normal tissues of different embryonic origins using the Mab of the invention are summarized in Table I.

TABLE I

Reactivity of the Mab of the invention with adult and fetal normal tissues (8-10 weeks)

Fetal tissues	Negative	: colon, skin, kidney
	Positive	: brain, stomach, thymus, lung, jejunum, liver
Adult tissues	Negative	: brain(2), lung(3), breast(3), stomach(4), duodenum(3), colon(3), liver(2), pancreas(1), kidney(4), urinary bladder(2), prostate(2), testis(2), endometrium(2), spleen(2), lymph nodes(2), skin(6), thymus(2), thyroid(2), meninges(2), skeletal muscle(1), choroid(2), retina(2).
	Positive	: synovia(3), ovary(1), Fallopian tube(1/4).

( ) between brackets the number of individuals from which tissues were obtained is indicated.

The studies carried out show that the recognized epitope has a highly specific tissue distribution. Among 10-12 weeks old fetal tissues, the Mab of the invention colors the inner coat of the blood vessels of stomach, jejunum, thymus and lung submucosa as well as the basement membrane of bronchial epithelium (pseudo glandular stage). With the exception of the basal portion of gastric glands, the older fetal tissues (up to 26 weeks) proved to be completely negative with the Mab of the invention, thus suggesting that the FN epitope identified by this reagent is subjected to a programmed expression during ontogenesis. In the adult samples the tissue distribution of said epitope, although different from the fetal condition, is much more restricted. Mab of the invention was found to color surface synovial cells, the inner coat of some ovarian vessels, isolated

areas of the bas ment membrane of celomic epithelium and, in a varying intensity, myometrium areas. Whereas normal tissues very seldom react with th Mab of the invention, as it is evident from Tabl II, the major part of the analyzed tumors contained remarkable levels of the epitope recognized by the antibody under test.

TABLE II

Kind of tumor	positive n./tested n.
Stomach adenocarcinoma	3/7
Pancreas "	0/3
Liver "	2/7
Colon "	2/10
Kidney "	4/8
Urinary bladder carcinoma	4/8
Prostata adenocarcinoma	1/5
Ovaries "	2/8
Endomethrium "	1/6
Skin - mixed type	3/8
Thyroid adenocarcinoma	1/8
Lung "	7/13
Skin and eyes melanomas	9/21
Breast adenocarcinoma	3/13
Brain - mixed type	4/13
Meningioma	13/13
Sarcomas of mixed type	3/15

The incidence of the positive coloration varied within a given type of tumor and no relationships with the differentiation degree (colon adenocarcinomas) nor with the tumor histiotype (breast tumors) were evidenced. The only exception is represented by fibromatous and endothelial meningiomas, which were found to be systematically positive for the carcinoma cases, in which both the normal tissue and the tumoral one from the same patient could be studied, the positive reaction with the Mab of the invention was found only in the neoplastic tissue. In almost all the samples which were positive with the Mab of the invention, the coloration was located in areas of varying extension of the interstice of the tumor, which surrounded or divided tumor cell nestes of variable measures, in which the coloration seldom delimited individual cells.

A frequent aspect of the tumors which were positive with the Mab of the invention was that the inner coat of the vessels was positive, whereas the one from the tested normal adult tissues was never positive, with the exception of some ovarian blood vessels.

A certain number of benign lesions (breast fibrosadenoma (4), mastopathias (2), gynecomastias (4), prostatic hypertrophy (5), urinary bladder and rectum polyps (4), intradermal nevi (14), angiomas (2), ovarial cystoadenomas (2), thyroid neurofibromas (1), chronic inflammatory lesions, hepatic cirrhosis (2), Echinococcus cysts (2), cutaneous scars (3) (10-30 days)) were tested with the Mab of the invention and all of them were found to be negative, with the exception of one breast adenofibroma which showed a coloration of the interstitial tissue in some areas.

From what stated above it is evident that the Mab of the present invention can be useful for diagnostic purposes for an early and sensitive diagnosis of tumors. To this purpose, the Mab can be employed in known immunoenzymatic, immunofluorescence techniques and the like, in which techniques the monoclonal antibody is labelled with enzymes, fluorescent compounds, luminescent compounds, radioactive compounds, ferromagnetic probes and the like. Particularly preferred is the enzyme labelling, in which case alcaline phosphatase, glucose oxidase, galactosidase, peroxidase and urease are usually employed. Enzyme labelling can be carried out according to any conventional methods, using for example glutaraldehyde, benzoquinone, periodate, protein A etc.

Once the labelled monoclonal antibody is obtained, analysis is performed using one of the many conventional immunoassay methods, for example those known as EIA or ELISA. Those skilled in the art, when the Mab of the invention is available, could easily develop a diagnostic kit or a suited method to detect transformed FN from bioptic samples. A positive response (i.e. due to th recognized presence of FN containing the ED-B sequence) is indicative of a tumoral or viral transformation occurred in the cells of the examined tissue.

Due to the specificity of the Mab of th invention to tumor-transformed cells, the antibody itself, suitably

bound to cytotoxic or anyway antitumoral medicaments can be used for therapeutic purposes in form of appropriate pharmaceutical compositions, which are a further object of th present invention.

The following non-limiting example illustrates the invention in more detail.

EXAMPLE

Preparation of the antigen.

The culture medium is drawn from culture confluent plates of WI38Va13 cells (SV40-transformed fibroblasts) cultured in the presence of FCS from which bovine FN had been removed. The medium is filtered on Millipore filters, added with NaN_3 and trasylol (final concentration 0,2% and 10 µ/ml respectively), then passed through a Sepharose 4B-conjugated gelatin chromatographic column. Up to 20 liters of medium are charged on a 150 cc columm.

Thereafter the column is washed with PBS (20 mM Na phosphate pH 7; 0,15 NaCl) containing 0,1% NaN_3, then with 4M NaCl in 50 nM Tris, pH 7, to remove the specifically bound proteins. Finally FN is eluted with 3M urea in PBS containing 0,1% NaN_3.

The whole procedure is carried out at room temperature, with a 200 ml/hr flow rate, recovering 10 ml fractions.

The fractions obtained washing the column with 3M urea and having an absorbance at 200 nm higher than 0,5 are combined in a pool and dialyzed against 20 volumes of PBS containing 0,1% NaN_3 and 2 mM PMSF.

Usually 5 mg of FN are obtained from 1 liter of medium. The FN obtained by this procedure turns out to be homogeneous in electrophoresis on polyacrylamide gels.

Mice immmnization and cell hybridization techniques

Balb/c mice are immunized with two intraperitoneal injections of purified FN, 50 µg, in about 100 µl of PBS emulsified with an equal volume of complete Freund adjuvant (DIFCO) and with three intraperitoneal injections containing 400 µg of antigen with no adjuvant. The two first injections are applied at 1 week intervals, the subsequent ones being administered daily during the 3 last days before the fusion.

Spleens are sterile removed and broken up into a single cell suspension using a stainless steel dense mesh net. Fusion is carried out by incubating 10 mouse splenocytes with 1/5 P3Ul plasmocytoma cells in 0.5 ml of polyethylene glycol (PEG 4000) with 50% DMEM-FCS, added dropwise in one minute. Then the mixture is shaken during 90 second at 37° C. After that, 20 ml of serum-free DMEM are added dropwise under stirring. The mixture is left to stand for 5 minutes; then 20 ml of DMEM containing 10% fetal calf serum (FCS) are added and the cells are centrifuged at 1000 x g for 5 min. The supernatant is removed and the cells are resuspended in 48 ml of DMEM-HAT (20% FCS, 1% hypoxanthine, 1% aminopterin, 0.2% thymidine, 0.2% glycine). The cells supension is then dispensed (1 ml per well) in two 24-well plates containing a monolayer of macrophages. 3 days after 1 ml of HAT medium is added to each well.

Said procedure is carried out for 2 weeks, at the end of which the growth of hybrids on the bottom of the 48 wells is observed.

Cloning

The presence of anti-FN antibodies in each well is checked by radioImmunoassay, then the cells contained in the "positive" wells are cloned.

The cells are removed from each well and resuspended in 10 ml of HAT medium, counted and diluted to obtain a suspension containing about 3 cells/ml.

About 200 µl of this dilution is dispensed per well in 96 wells already containing mouse peritoneal macro phages.

About 2 weeks after, the medium from those wells having at least 1/3 surface covered with cells is assayed for anti-FN antibodies by radioimmunoassay.

Radioimmunoassay

Radioimmunoassay is performed accordingly to the suitably modified method described by Accolla and Celada [Accolla, R.S., Celada, F. (1978) Eur. J. Immunol. 8, 688].

96-well polyvinyl plates ("Microvil" 24 A m.cat. 968 Pbi) are used. Polyvinyl is a material characterized by having binding sites for proteins. FN (at a concentration of 100 µg/ml) purified according to the above described method is incubated in the wells at room temperature. 2 hours after the protein is removed from the wells, which are filled with PBS containing 1% bovine serum albumin (200 µl per well) to take up unoccupied binding sites. After two further hours the albumin solution is removed and the wells are washed with PBS. Then the culture media of the hybrids whose capability to release anti-FN antibodies is to be checked are added (100 µl per well). After 2 hours more said media are removed off and the wells are washed again with PBS. At this point, mouse anti-FAB antibodies induced in rabbits, labelled with I are added, 100 µl per well, and incubated at room temperature for 2 hours. Subsequently the anti-PBS radioactive antibodies are added, finally each well is placed into a numbered test tube. Radioimmunoassay is carried out by means of a gamma-counter (Packard) for a period of 10 minutes for each tube.

Determination of the antibody specificity

Among the hybridomas prepared by the described method, a clone was obtained which releases antibodies which react with Wi38Va13 FN and do not react with FN from plasma or normal cells. This specificity was assayed by two different methods:

A) Radioimmunoassay.

FNs purified from Wi38Va13 cells, from normal fibroblasts or from plasma culture media were used for the radioimmunoassay, according to the above described method.

The antibody under test gave counts with positive values only with FN from Wi38Va13 cells.

B) Western blot.

FNs purified from Wi38Va13 cells, from normal fibroblasts or from human plasma culture media was caused to migrate on a SDS polyacrylamide gel according to the method by Laemmli [Laemmli, U.K. (1970) Nature, 227, 680-685].

After the electrophoresis, the proteins were transferred on a nitrocellulose filter (Western blot) according to the method described by Towbin et al. (1979), Proc. Natl. Acad. Scl., 76, 4350-4354.

After that, filters were washed with 6 M urea in distilled water for 10 minutes, to remove SDS. After thorough washing with distilled water, then with TBS (10 mM Tris-HCl pH 7,4; 0,15 M NaCl), the filters were incubated for 2 hours with anti-FN monoclonal antibodies containing 0,05% Tween 20 (Merck). The anti-FN antibodies excess was removed by washing with TBS (5 changes, 30 minutes total); then filters were incubated for 2 hours with a second antibody conjugated with mouse anti-Ig peroxidase (Dako, Copenhagen, Denmark) diluted 1/1000 in TBS, 3% BSA, 0,05% Tween 20.

After repeated washings with TBS (5 changes, 30 minutes, total) and two washings with TB (10 nM Tris-HCl pH 6,8), filters were incubated with a substrate solution to visualize the antigen bands. Substrate was prepared immediately before use, as followings:

20 ml of TB, 6,63 µl of 30% H_2O, 5 ml of a 0.3% (w/v) 4-chloro-1-naphthol solution (Merck; Darmstad, West Germany) in methanol.

4-Chloro-1-naphthol solution can be preserved for one month at 4°C. The reaction is quenched by washing filters with distilled water after 10-120 minutes (the time depending on the antigen amounts and on the activity of the first antibody). Filters are dryed between two paper filters and photographed. By this technique, the only protein which can react with the antibody under test is FN from Wi38Va13 cells.

Localization of the antigenic determinant recognized by the Mab

The Western blot technique was used to localize the epitope recognized by this antibody.

FNs from Wi38Va13 cells, from normal fibroblasts and from plasma were thermolysin digested. Each type of FN was purified, dialyzed in 25 mM Tris-HCl, pH 7,6, against 0.5 mM EDTA, 50 mM NaCl, 2.5 mM CaI_2 and incubated with thermolysin (5 µm/mg FN) for 4 hours at 22°C. EDTA was added to a final 5 mM concentration to quench the reaction. The obtained digests were caused to migrate on a SDS polyacrylamide gel, according to the method by Laemmli, U.K. (1970) Nature, 227, 680-685 to separate the protein fragments depending on the molecular weight thereof. Then peptides were transferred on a nitrocellulose sheet, by the Western blot technique. Different filters were prepared with the same sample series, to test some of them with Mabs whose antigenic determinants have a known location on the FN molecule, whereas on one of these filters the Mab according to the invention was tested, which is hereinafter defined as BC-1. [Fig. 1 - Model of the domain structure of a subunit of human FN. Big white arrows indicate the regions of variability due to alternative splicing of the FN mRNA precursor. Long white arrows indicate the sites in which the epitopes recognized by the various Mabs are located. The figure also shows the three types of internal homologies, the macromolecules interacting with the various FN domains and the possible isoforms generated by different splicing models. Fig. 2 -Electrophoresis on SDS polyacrylammide 4-18%-gel of plasma FN (P); FN from Wi38 normal human cells (N) and from Wi38Va13, SV40-transformed human fibroblasts (T) digested by thermolysin (5 µg/mg of FN for 2 hours at 22°C). The values on the left are the molecular masses of standards in kDa. The values on the right are the molecular masses in kDa of the four major fragments present only in the thermolysin digest of FN from transformed cells and of the 110-kDa fragment, which constitutes domain 4. Immunoblots of similar gels, respectively colored with BC-1; IST-4; 3E3 and IST-9 are also shown. Evidenced are the two forms of domain 4, plus or without the ED-B sequence.]

This antibody strongly reacts with a 120 kDa fragment which represents the cell binding form containing the ED-B sequence [Zardi, L., Carnemolla, B., Siri, A., Petersen, T.E., Paolella, G., Sebastio, G., Baralle, F.E. (1987) EMBO J., 6, 2337-2342] but does not react with the 110 kDa fragment, which represents the cell binding domain without the ED-B sequence (figure 1). This datum indicates that the epitope recognized by the BC-1 antibody is contained in the ED-B sequence.

In order to prove further this result, the two 120 and 110 kDa fragments were purified and further digested.

120 and 110 kDa fragments to FN (figure 1) were purified from a FN thermolysin digest (6 µg/mg of FN for 2 hours at 22°C) by a hydroyapatite (DNA-Grade, BioRad Laboratories, Richmond, Ca) chromatographic column as previously described [Borsi, L., Castellani, P., Balza, E., Siri, A., Pellecchia, C., De Scalzi, F., Zardi, L. (1986) Anal. Biochem., 155, 335-345]. Complete separation of the 120 kDa from the 110 kDa fragment was

achieved using a DEAE-cellulose (DE + 52, Whatman, Maidstone, UK) chromatographic column. Both the 120 and 110 kDa fragments were digested with thermolysin.

110 kDa fragment is resistant to thermolysin whereas the presenc of the ED-B sequ nce in the 120 kDa fragment introduces a thermolysin-sensitive site in this molecul [Zardi, L., Carnemolla, B., Siri, A., Petersen, T.E., Paolella, G., Sebastio, G., Baralle, F.E. (1987) EMBO J., 6. 2337-2342]. [Figure 3 - Schematic representation of thermolysin digestion of the 110 kDa fragment (domain 4 without ED-B) and of the 120 kDa fragment (domain 4 plus the ED-B sequence). The cut by means of S-cyanilation of the 85 kDa fragment obtained in the domain 4 (120 kDa) is schematically shown. The epitopes recognized by Mabs 3E3, IST-4 and BC-1 are also indicated. The numbers on the left show the corresponding columns in the gels and immunoblots reported in figure 4. Figure 4 - On the left is reported the electrophoresis on SDS polyacrylamide gel 4-18% of the following examples: purified 110 kDa fragment (lane 1); purified 120 kDa fragment containing the ED-B sequence (lane 2); purified 110 kDa fragment digested with thermolysin (lane 3); 120 kDa fragment digested with thermolysin (lane 4); purified 85 kDa fragment from the thermolysin digest of 120 kDa fragment (lane 5) and the 85 kDa fragment cut by S-cyanilation (lane 6). On the right the immunoblots of similar gels colored using Mabs BC-1, IST-4 and 3E3, respectively, are reported.]. Thus, thermolysin digestion of domain 4 from 120 kDa gives two fragments: a 35 kDa and an 85 kDa fragment.

These fragments were tested after electrophoresis and blotting with Mab BC-1 and the obtained results proved that the only 85 kDa fragment reacts with Mab BC-1.

The determination of the amino acid sequence proved that 35 kDa fragment contains 7 amino acids of the ED-B sequence in its amino terminal portion; the 85 kDa fragment contains the ED-B sequence almost complete in its carboxy terminal portion.

The 85 kDa fragment was purified and its carboxy terminal portion was cut by S-cyanilation [Sekiguchi, K., Hakomori, S. (1983) Biochemistry, 22, 1415-1422] to produce a 65 kDa fragment which cannot any longer react with Mab BC-1 (see Figures 3 and 4).

Claims

1. Monoclonal antibody specific to the ED-B sequence of fibronectin from transformed cells, obtained from the hybridoma deposited at the European Collection of Animal Cell Cultures (ECACC) on April 21, 1988, under the No. 88042101.

2. Hybridoma ECACC 88042101.

3. A method for the diagnosis of tumors in bioptic samples, characterized in that said samples are tested with the monoclonal antibody of claim 1 or with the same antibody, suitably labelled, for the presence of transformed fibronectin.

4. The method according to claim 3, which uses the monoclonal antibody labelled with enzymes, fluorescent compounds, luminescent compounds, ferromagnetic probes or radioactive compounds.

5. A kit for the diagnosis of tumors, comprising the monoclonal antibody of claim 1 or the same labelled antibody and other possible reagents conventionally used in immunodiagnostic methods.

Claims for the following Contracting States:ES, GR.

1. A method for the diagnosis of tumors in bioptic samples, characterized in that said samples are tested with a monoclonal antibody specific to the ED-B sequence of fibronectin from transformed cells, obtained from the hybridoma deposited at the European Collection of Animal Cell Cultures (ECACC) on April 21, 1988, under the No. 88042101, or with the same antibody, suitably labelled, for the presence of transformed fibronectin.

2. The method according to claim 1, which uses the monoclonal antibody labelled with enzymes, fluorescent compounds, luminescent compounds, ferromagnetic probes or radioactive compounds.

FIGURA 1

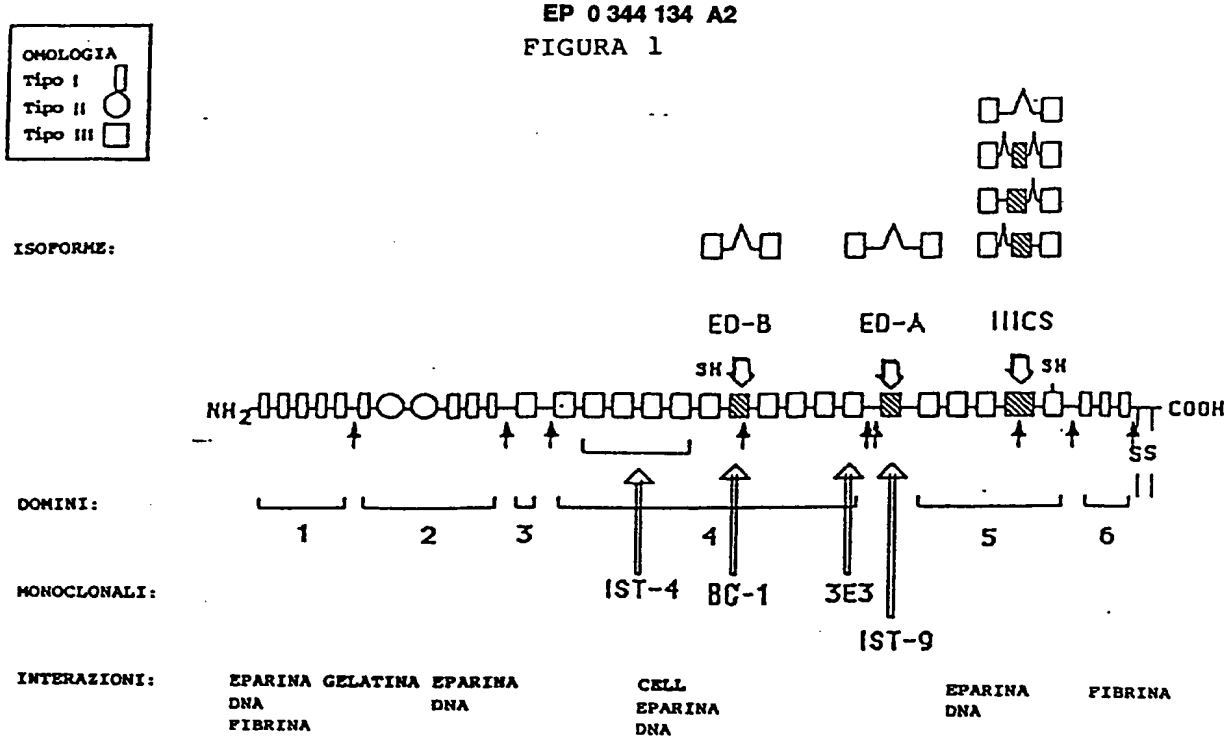

FIGURA 2

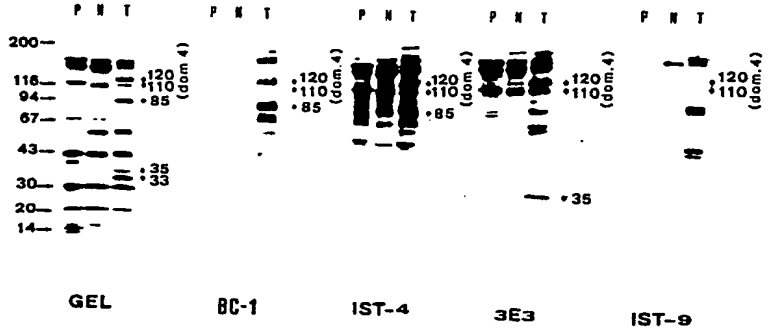

FIGURA 3

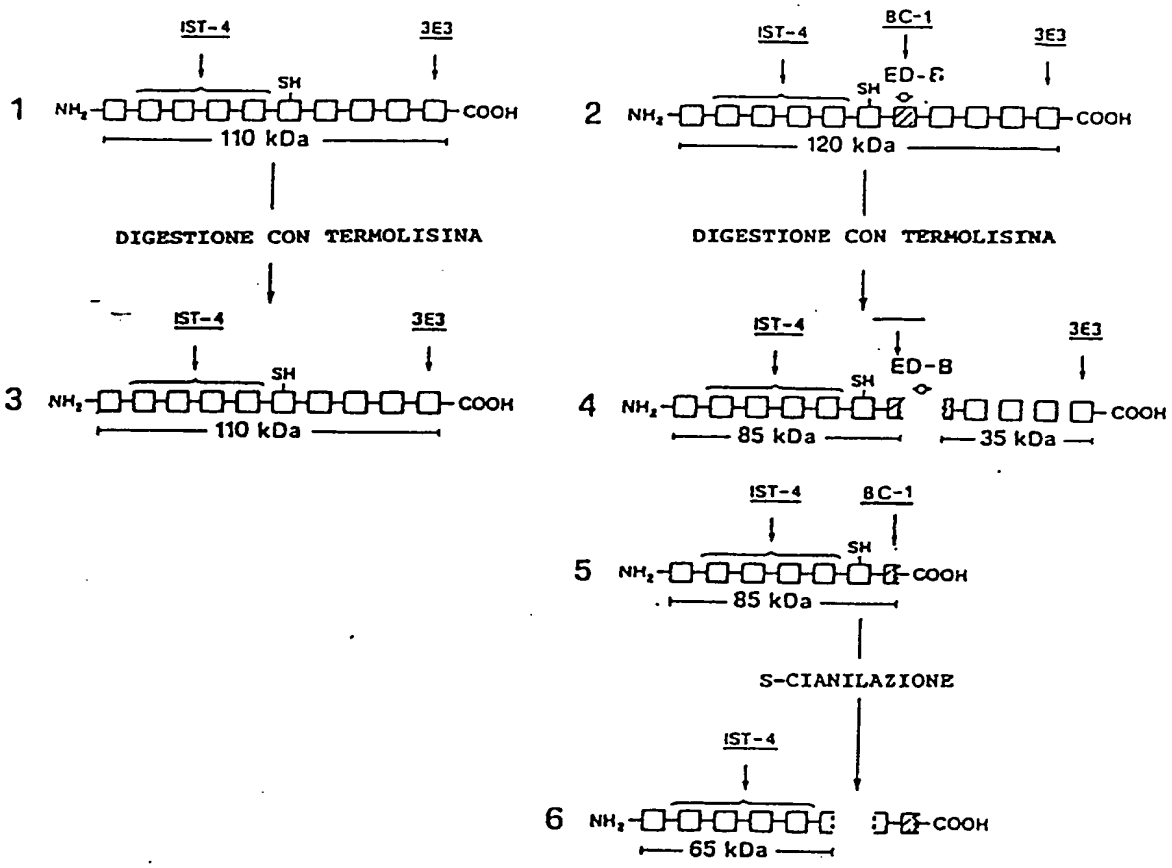

FIGURA 4